A loop-mediated isothermal amplification method for rapid direct detection and differentiation of non-pathogenic and verocytotoxigenic *Escherichia coli* in beef and bovine faeces

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Running Title: Multiplex LAMP assay for *E. coli* and VTEC

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Abstract

Aims: To develop a multiplex loop-mediated isothermal amplification (LAMP) assay capable of quantifying *Escherichia coli* and differentiating verocytotoxigenic *E. coli* (VTEC).

Methods and Results: Primer sets were selected to amplify the *phoA* gene (all *E. coli* strains) and *stx1* and/or *stx2* genes (VTEC strains only). LAMP calibration curves demonstrated good quantification capability compared to conventional culture. The limits of detection 50% (LOD_{50}) of the multiplex LAMP assay were 2.8 (95% CI 2.4-3.3), 3.2 (95% CI 2.5-3.9) and 2.8-3.2 (95% CI 2.1-3.5) log CFU g^{-1} for the *phoA*, *stx1* and *stx2 genes,* respectively. When validated by testing retail beef and bovine faeces samples, good correlation between *E. coli* counts indicated by the LAMP assay and culture was observed, however false negative LAMP assay results were obtained for 12.5-14.7% of samples.

Conclusions: A rapid, multiplex LAMP assay for direct quantitation of *E. coli* and specific detection of VTEC in beef and faeces was successfully developed. Further optimisation of the assay would be needed to improve detection sensitivity.

Significance and Impact of the Study: The multiplex LAMP assay represents a rapid alternative to culture for monitoring *E. coli* levels on beef for hygiene monitoring purposes, and, potentially, a method for detection of VTEC in beef and faeces.

Keywords: *Escherichia coli* (all pathogenic types), Detection, Beef, Rapid methods, Loop-mediated isothermal amplification (LAMP)

Introduction

Escherichia coli colonises the intestines and faeces of humans and animals and is among the first bacterial species to colonize the intestine during infancy (Tenaillon et al. 2010). Detection of *E. coli,* generically, is used as a hygiene indicator in food and water. In the beef industry specifically, enumeration of *E. coli* is carried out to confirm hygienic conditions during slaughtering and assess quality of meat (Barros et al. 2007). According to EU

Regulation EC No. 1441/2007, on the microbiological criteria for foodstuffs, the *E. coli* limits for minced meat and meat preparations are 50-500 and 500-5000 CFU g^{-1}, respectively (European Commission 2007). Pathogenic types of *E. coli* also occur on beef, and, in particular, verotoxigenic *E. coli* (VTEC) are zoonotic agents that cause severe diseases (Marrs et al. 2005; Klein et al. 2006; Tenaillon et al. 2010) and are responsible for many foodborne outbreaks worldwide (Michino et al. 1999; Food Standards Agency 2011). The foods most frequently implicated in VTEC outbreaks are apple juice, raw milk, raw beef, cheese and fresh produce (Wang et al. 2012ab). *E. coli* O157:H7 is the most common serogroup associated with illnesses and deaths in humans (Scallan et al. 2011), with clinical manifestations ranging from abdominal pain and diarrhoea, to haemorrhagic colitis and potentially fatal haemolytic-uremic syndrome (Food Standards Agency 2011; Ravan et al. 2016). The digestive tract of ruminants is considered to be the main source of this pathogen, and foods of bovine and ovine origin are frequently reported as vehicles for human VTEC infections (European Food Safety Authority 2010). Ruminant faecal contamination of crops and water systems, as well as direct contact with animals, has also been established as a cause of infection (Wise, 2009). In the USA, the Centre for Disease Control and Prevention estimated that *E. coli* O157:H7 infections result in approximately 93,000 illnesses, 2100 hospitalizations and 20 deaths each year (Scallan et al. 2011). However, several other serogroups have been linked to outbreaks and sporadic cases (European Food Safety Authority 2014). For example, the 2011 sprout outbreak in Germany that led to 4,075 cases and 50 deaths was attributed to serotype O104:H4 (Buchholz et al. 2011). As a result of the occurrence of foodborne outbreaks of non-O157 VTEC on an international level, the USA requires routine verification testing for serogroups of the most prevalent non-O157 serogroups (i.e. O26, O45, O103, O111, O121 and O145). These six non-O157 serogroups share many virulence characteristics with *E. coli* O157:H7, and both O157 and non-O157 serogroups have been shown to have an infection dose of <100 cells (United States Department of Agriculture 2011). All VTEC serotypes harbour genes that can produce at least one verotoxin (Stx1 and/or Stx2), which constitute the main virulence factors of VTEC

(Thorpe 2004). The pathogenicity of VTECs has also been linked to a number of virulence factors other than verotoxins, e.g. intimin (encoded by *eae* gene) responsible for attaching and effacing of the pathogen to the gut epithelial cells (European Food Safety Authority 2013). The low infective dose of VTECs, in conjunction with the severity of the resulting illness, necessitates the use of rapid and reliable detection methods that can be used at processing level to monitor VTEC contamination, in order to protect consumers.

Conventional culture methods, although time consuming, can readily identify *E. coli* O157:H7 based on their inability to ferment sorbitol when cultured in sorbitol-containing selective media. However, due to the lack of a unique phenotypic characteristic for other serogroups, selective culture-based methods to detect the major non-O157 VTECs are not well established (Noll et al. 2015). Enzyme immunoassays have been used for the detection of verotoxins and some VTEC serogroups. However, false-positive results have been reported and high levels of the target pathogenic bacteria must be present (Chapman et al. 2001; Gould et al. 2009). Molecular-based methods have also been employed in the detection of *E. coli* and are viewed as specific, accurate and sensitive. PCR or qPCR methods have been widely used for *in vitro* amplification of gene targets in order to achieve pathogen detection. These PCR methods are based on the use of different temperatures (to achieve denaturation, annealing and DNA extension) and, although still a relevant and important diagnostic tool, PCR can be time consuming and can require the use of expensive thermocyclers (Oh et al. 2016), limiting their accessibility for food industry use. In order to replace the expensive, complex and time-consuming thermal cycling amplification required by conventional PCR, isothermal DNA amplification methods have been developed. One such method, loop-mediated isothermal amplification (LAMP), has gained increased attention for a number of important reasons. Firstly, LAMP does not require a denatured template as it uses *Bst* DNA polymerase with auto-cycling strand displacement activity. Secondly, test duration is only 60 min due to use of four to six specially designed primers which amplify DNA targets, and, thirdly, one specific amplification temperature can be used (Notomi et al. 2000). Simply put, the isothermal nature of LAMP significantly simplifies the

detection process and it does not require expensive complex instrumentation; it can even be performed using heating blocks or water baths. Moreover, compared to conventional PCR, LAMP positive results can be more easily detected via visual observation of turbidity changes (Mori et al. 2001), simplifying detection even further.

Several LAMP assays targeting generic *E. coli*, *E. coli* O157 or different VTEC virulence genes have been developed to date (Maruyama et al. 2003; Hill et al. 2008; Kouguchi et al. 2010; Wang et al. 2012ab; Ravan et al. 2016). However, to the best of our knowledge, no LAMP assays able to detect an *E. coli* specific gene and verotoxin producing genes simultaneously, as well as distinguish between generic *E. coli* and VTEC, are currently available. A method capable of detecting both *E. coli* and VTEC simultaneously would provide the beef industry with a tool that would allow the simultaneous monitoring of hygienic status/quality and VTEC contamination of beef, which would facilitate day-to-day decision-making. Therefore, this proof-of-concept study had two aims. Firstly, to develop a rapid and sensitive LAMP assay for the multiplex detection of generic *E. coli* and VTEC strains specifically, targeting *phoA* and *stx1/stx2* genes, respectively, without the need for culture enrichment or DNA purification. Secondly, to evaluate the detection limit and quantification capability of the novel LAMP assay using artificially- and naturally-contaminated beef and bovine faeces samples.

Materials and methods

Bacterial strains used, culture conditions and DNA extraction

Fifty-eight bacterial cultures were used during this study, comprised of non-pathogenic *E. coli* (n=5), VTEC strains belonging to serogroups O26, O145, and O157 (n=7), and a range of non-target foodborne pathogenic and spoilage bacteria (n=46). These are listed in more detail in Table 1. All bacterial strains were cultured at 37°C overnight in Brain heart infusion (BHI) broth (Oxoid, UK). *Campylobacter* strains were grown under microaerobic conditions

(85% N_2, 10% CO_2, and 5% O_2) at 37°C. A heating method was used to extract DNA from all bacterial strains. Briefly, for each strain, 1 ml of broth culture at stationary phase (approximately 10^9 CFU ml^{-1}) was centrifuged at 16,000 × g for 5 min. The pellet was resuspended and washed twice in molecular grade DNAase-free sterile water, then heated at 99°C for 15 min in a heating block (Stuart Scientific, UK), before being centrifuged at 16,000 × g for 5 min to sediment cell debris. The supernatant was used as a template for LAMP assays. Aliquots of 2 µl of each template were tested by LAMP, repeated three times. The CFU ml^{-1} of each bacterial culture was determined by a conventional pour plate method using Rapid 2 agar (BioRad, UK).

LAMP primer set design and selection

Each LAMP primer set used in this study consisted of two outer (F3, B3), two inner (FIP, BIP) and two loop primers (Loop F, Loop B), which recognised eight different regions of the gene target and were designed by *Lamp Designer* Software (http://www.optigene.co.uk/lamp-designer/). Five primer sets were designed for detection of the *phoA* gene target (GenBank accession no. M29667). Three primer sets for each of the VTEC gene targets (*stx1 and stx2*) were selected from previous publications (Hara-Kudo et al. 2007; Wang et al. 2012b; Dong et al. 2014). The primer sets for the *stx1* and *stx2* genes described in the study of Dong et al. (2014) were found to be the most suitable ones. Table 2 lists the sequences of the oligonucleotide primer sets ultimately selected for use in the optimised LAMP assay, on the basis that the amplification products had unique annealing temperature values.

LAMP conditions and multiplexing

LAMP was carried out in a final reaction volume of 25 µl. The reaction mixture contained 15 µl of isothermal Master Mix ISO 001 (OptiGene Limited, UK) containing *Geobacillus* species DNA polymerase, thermostable inorganic pyrophosphatase, $MgCl_2$, deoxynucleoside triphosphates and double-stranded DNA dye. Also, the reaction contained a primer mix

consisting of six primers for each of the three gene targets (F3 and B3 primers at 2 x 10^{-7} mol l^{-1}, FIP and BIP primers at 8 x 10^{-7} mol l^{-1}, and LF and LB primers at 4 x 10^{-7} mol l^{-1}), and 2 µl DNA. The LAMP assay was run at 65°C in a real-time fluorometer (Genie II, OptiGene Limited, UK) for 30 min. The simultaneous detection of *phoA*, *stx1* and *stx2* (multiplexing) was based on the different annealing temperatures of the three amplicons as determined by annealing curve analysis. Amplicon annealing curve analysis was performed in the Genie II fluorometer after the completion of the amplification phase by heating to 98°C and subsequent gradual cooling to 80°C at a rate of 0.05°C sec^{-1}.

Conventional cultural enumeration of *E. coli*

Samples of beef (10 g) or faeces (1 g) were placed in a sterile stomacher bag and a 10^{-1} dilution prepared by the addition of an appropriate volume of Maximum recovery diluent (MRD, Oxoid, UK). The sample was homogenised in a stomacher for 1 min at 320 rpm before further decimal dilutions were prepared in MRD, as required. *E. coli* were enumerated by pour plating 1 ml of each dilution with Rapid 2 agar (Bio-Rad, UK) and incubating at 37 °C for 24 h. Each sample was plated in duplicate. This culture method permits the enumeration of both VTEC and non-VTEC, but does not allow differentiation of the two types, and is the culture approach used by the beef processing company that funded this study. The method has been validated by AFNOR certification as an alternative to ISO 1664-2 (ISO 2001), which is why it was chosen as the comparator method during this study.

LAMP assay specificity

For LAMP specificity checks, DNA from 58 bacterial strains was prepared by heating at 99°C for 15 min, as described above. Two microliter aliquots of each DNA template were subjected to LAMP amplification using *phoA* primer set only. The presence or absence of the amplicon was recorded for each bacterial strain. Specificity of *stx1* and *stx2* primer sets has previously been confirmed by Dong et al. (2014), so was not retested during this study.

LAMP assay sensitivity assessment with pure *E. coli* cultures

LAMP sensitivity (expressed as Limit of detection 50% or LOD_{50}) was determined by testing 10-fold serial dilutions of VTEC and non-VTEC strains. Briefly, each strain was grown separately in 10 ml BHI broth and incubated at 37°C overnight to reach the stationary phase. Ten-fold serial dilutions were prepared and aliquots (1 ml) of each dilution were used to prepare DNA templates by heating as described above. The cell number was determined by conventional plate pour plating using Rapid 2 agar. Triplicate aliquots (2 µl) of each DNA template were tested by LAMP.

Determination of LAMP assay detection sensitivity using artificially contaminated beef and bovine faeces

Gamma-irradiated (10 kGy) samples of beef (25 g) or bovine faeces (1 g) were inoculated with 10-fold serial dilutions of individual overnight VTEC cultures (VSD 397, EDL 933, CDC 99-311, CDC 03-3014), or a non-VTEC strain cocktail (NCTC 12900, BAA 1427, BAA 1428, BAA 4129, K12, ER 2738), resulting in contamination levels of between 10^7 and 10^0 CFU g^{-1}. An additional beef or faeces sample was included as the non-inoculated control. The samples were placed in a sterile stomacher bag and a 10^{-1} dilution prepared in MRD by homogenizing in a stomacher for 1 min at 320 rpm. Aliquots (1 ml) of the homogenates were centrifuged at 16,000 × *g* for 5 min, and pellets were resuspended in 100 µl DNAase free water and washed twice. The samples were heated at 99°C for 15 min before centrifugation at 16,000 × *g* for 5 min to sediment cell debris. The supernatants (2 µl) were used as DNA template for the LAMP assay, which was performed in triplicate for each sample. The presence or absence of *phoA, stx1* and *stx2* amplicons was recorded for each spiked sample. Aliquots (1 ml) of each homogenate were also used for conventional enumeration of *E. coli* on Rapid 2 agar, as described above.

Validation of LAMP assay by testing naturally contaminated beef and bovine faeces

In order to validate the optimised multiplex LAMP assay for detection of *E. coli* and VTEC, 32 fresh beef samples, purchased from local butcher shops and supermarkets, and 34 bovine faeces (archived samples from a previous study that had been stored at -80^{o}C) were tested. Beef and faeces samples were prepared as described above for artificially spiked samples, and crude DNA extracts were used directly for the LAMP assay, without any further purification. The presence or absence of *phoA, stx1* and *stx2* amplicons was recorded for each sample.

Statistical analysis

Experiments were performed at least three times. The 50 % endpoint Limit of Detection (LOD_{50}), the absolute performance efficacy and associated uncertainties of the detection assay were calculated using the Spearmann-Kärber method (Association of Official Analytical Chemists 2006), for the pure cultures and artificially inoculated beef and faeces. Calibration curves to quantify *E. coli* in pure culture, beef and bovine faeces were produced by plotting T_t values versus log CFU ml^{-1} or g^{-1}. The regression line equations of the calibration curves were used to quantify *E. coli* detected by the LAMP assay. The *E. coli* levels present in beef and faeces samples were calculated by inserting the T_t values obtained into the appropriate equation. In order to validate the calibration curve equations, the predicted counts were plotted against the *E.coli* levels as determined by the conventional method and the correlation (R^2) values determined using Microsoft Excel software.

Results

Specificity of LAMP *phoA* primers

The LAMP assay was designed for the detection of non-pathogenic *E. coli* targeting the *phoA* gene and VTEC targeting the *stx1* and *stx2* genes. In total, 58 bacterial strains were used to determine the specificity of the *phoA* LAMP assay. The assay showed 100%

specificity for *E. coli* with no false-positive or false-negative results with strains of any of the other bacterial genera tested, i.e. all *E. coli* strains were positive for the *phoA* gene and non-*E. coli* showed no amplification. Table 1 presents results obtained for the 58 bacterial strains tested by the *phoA* LAMP assay. The specificity of the *stx1* and *stx2* primer sets had previously been assessed (Dong et al. 2014), so was not assessed again as part of the current study.

LAMP optimisation and multiplexing

This study aimed to develop an assay that could detect and quantify *E. coli* generically and also offer additional information on the presence of VTEC. Upon detection of target DNA(s), the Genie II instrument displays amplification signals (*Tt*) as well an annealing temperature value (*Tm*) for each reaction. *Tm* is unique for each LAMP primer set used, therefore multiplexing of *phoA*, *stx1* and *stx2* genes was based on the premise that sufficiently different *Tm* values would allow separation of each amplified product and thus would result in distinct annealing peaks after annealing curve analysis. Several preliminary trials were conducted in order to identify suitable primer sets (six primers for each set) for all three gene targets. Each primer set for each of the gene targets was first tested individually in order to determine its specific annealing temperature. Figure 1 presents typical examples of peaks after annealing curve analysis of the different primer sets tested. Primer sets for all three gene targets with similar *Tm* values were excluded, as they would not allow the generation of distinct peaks. The primers selected for the final LAMP assay had a *Tm* for *phoA* that ranged from 90.0–90.6°C, a *Tm* for *stx1* that ranged from 86.0-86.3°C and a *Tm* for *stx2* that ranged from 88.0-88.3°C, thus allowing separation of distinct peaks (as illustrated in Fig. 2B). Subsequently, trials were conducted by combining the three selected primer sets for the three target genes tested simultaneously. As each LAMP primer set can have a different amplification efficiency, multiple combinations of different concentrations of each primer set were tested. The primer quantity per reaction that gave the best results was 2 µl for *phoA*, 2 µl for *stx1* and 1.5 µl for *stx2* (primer concentrations are mentioned in the LAMP conditions

and multiplexing section). Figure 2 illustrates the annealing curve analysis of the three amplicons for beef samples positive for generic *E. coli* or positive for VTEC. Annealing curve analysis shows the generation of one or three distinct peaks, which, therefore, permits discrimination between presence of generic *E. coli* and VTEC specifically, as well as simultaneous detection of the three targets.

LAMP sensitivity testing with pure *E. coli* cultures, and spiked beef and bovine faeces

The sensitivity of the assay was first tested with individual *E. coli* strains (non-VTEC and VTECs) as pure cultures. Ten-fold dilutions of the individual strains were prepared and tested after DNA extraction. In total, six non-VTEC strains (NCTC 12900, BAA 1427, BAA 1428, BAA 4129, K12, ER 2738) and four VTEC (VSD 397, EDL 933, CDC 99-311, CDC 03-3014) were tested. The LOD_{50} values ranged from 2.5-3.5, 2.5-2.8, 2.5-3.2 log CFU ml^{-1} for the *phoA*, *stx1* and *stx2* gene target, respectively (Table 3). The R^2 values for quantification of *E. coli* were found to be 0.824-0.999, 0.968-0.996 and 0.779-0.921 for the *phoA*, *stx1* and *stx2*, respectively (data not presented).

Figure 3 shows a typical LAMP amplification graph and the corresponding calibration curve, along with the derived quantification equation and R^2 values, for the *phoA* gene detection in artificially spiked beef samples. Table 4 presents in detail the LAMP analytical sensitivity after testing 10-fold serial dilutions of the VTEC and non-VTEC cocktails in beef and faeces. For the *phoA* target, the LOD_{50} was 2.8 log CFU g^{-1} for both beef and faeces. For the *stx1* target, the detection limit for both beef and faeces was found to be 3.2 log CFU g^{-1}. The *stx2* target showed a higher detection limit in beef (3.5 log CFU g^{-1}) compared to faeces (2.8 log CFU g^{-1}), although *stx2* detection in faeces showed a wider 95% confidence interval. Calibrations curves for each sample matrix and each gene target were generated. Based on these, quantification equations and R^2 values were calculated (Table 5). For beef samples, R^2 values for *phoA*, *stx1* and *stx2* were between 0.932 and 0.989 showing high correlation with counts obtained by culture on Rapid 2 agar, i.e. good quantification potential. Similarly, the R^2 values for faeces samples for *phoA* and *stx1,* ranging from 0.932-0.955,

indicated good correlation with culture results; however, the R^2 for the *stx2* target was lower at 0.763, although this still represents good correlation.

Validation of the optimised LAMP assay by testing naturally contaminated beef and bovine faeces samples

The equations generated from the calibration curves for artificially inoculated beef and faeces (Table 5) were used to predict the levels of *E. coli* present in naturally contaminated retail beef and bovine faeces samples containing unknown levels of *E. coli* by inputting the *Tt* value obtained after LAMP into the appropriate equation. To validate the performance of the optimised LAMP assay, beef and bovine faeces were sourced and tested directly following DNA extraction by heating only. Conventional enumeration of *E. coli* by pour plating with Rapid 2 agar was carried out in parallel for comparison purposes. A total of 34 bovine faeces samples were tested, 12 (35.2%) of which tested negative for *E. coli* presence by the conventional culture method (detection limit = 10 CFU g^{-1}). Five samples (14.7%) tested positive by the conventional culture method, with *E. coli* counts ranging from 2.6-2.9 log CFU g^{-1}, but no amplification was observed with the LAMP assay; which is consistent with differences in the detection sensitivities of the two methods. The rest of the faeces samples (n=17, 50%) tested positive by both culture and LAMP methods. It is noteworthy that for all the *E. coli* positive samples detected only one peak corresponding to the *phoA* gene was observed in the annealing curve analysis, indicating that *E. coli* detected in the faeces were not harbouring the *stx1* or *stx2* genes or, alternatively, that VTEC were present at levels below the LAMP detection limit. Fig. 4 demonstrates the good correlation (R^2=0.8514) between the *E. coli* levels predicted by LAMP (using the *phoA* equation for spiked faeces) and *E. coli* levels indicated by the conventional plate counts for faeces.

Thirty-two retail beef samples were also tested, 24 (75%) of which were found to be negative for *E. coli* as determined by the conventional culture method. Four beef samples (12.5%) tested positive for *E. coli* by culture, with levels ranging from 1.00–2.95 log CFU g^{-1}, but did not show any amplification signal by the LAMP assay. A further four samples (12.5%)

tested positive by both the conventional culture method and LAMP assay with levels ranging from 3.00-4.04 log CFU g^{-1}. As was the case when bovine faeces was tested, all the LAMP positive beef samples yielded only the *phoA* gene peak indicating that the *E. coli* detected did not harbour *stx1* or *stx2* genes, or, alternatively, that VTEC were present at levels below the LAMP detection limit. A good correlation (R^2=0.76) was observed between the conventional plate counts and the *E. coli* counts predicted by the LAMP assay for these samples using the *phoA* equation for spiked beef (Fig. 5).

Discussion

Several different LAMP assays for non-pathogenic and pathogenic strains of *E. coli* have been developed previously (Yano et al. 2007; Hara-Kudo et al. 2007, Wang et al. 2009; Zhao et al. 2010). In a study by Hill et al. (2008) a LAMP assay for urine samples was developed targeting common strains of *E. coli*. The *malB* gene was used for detection, as it is conserved across different *E. coli* lineages; however this gene has also been found in *Shigella* spp. The use of *malB* gene as an assay target would have restricted the application of the LAMP method developed during this study as it would not have allowed the specific detection of *E. coli* in beef and faecal material. In contrast, the alkaline phosphatase gene (*phoA*) is present in all *E. coli* strains and has already been used in PCR-based methods for the detection of *E. coli* strains (Chang et al. 1986; Kong et al. 1995 and 1999; Thong et al. 2011; Ho et al. 2013; Teh et al. 2014), demonstrating high specificity. Therefore, the *phoA* gene was chosen in this study as a *target for detection of all E. coli (both non-VTEC and VTEC). Additionally, stx1 and* stx2 genes were chosen as targets for detection at they are considered the main virulence factors of VTEC (Thorpe 2004). The LAMP primer sets selected for the three gene targets on the basis of evaluations carried out during this study did not show overlapping *Tm* values, which, therefore, permitted the detection and differentiation of *E. coli* generally and VTEC specifically. Upon detection of *E. coli* in a sample an individual peak corresponding to *phoA* will appear in the annealing curve

analysis. Upon detection of VTEC in a sample, two or three peaks corresponding to *phoA*, *stx1* and/or *stx2* will be obtained (Fig. 2).

During this proof-of-concept study a novel multiplex LAMP assay was successfully developed for the rapid detection (<40 min), differentiation and quantification of *E. coli* generally and VTEC specifically in beef and bovine faeces samples, without the need for a culture enrichment step or DNA purification. This LAMP assay could potentially provide the beef industry with information on the presence of VTEC as well as hygienic status/quality of beef by detection of *E. coli* contamination more generally. The multiplex LAMP assay detected both pathogenic and non-pathogenic types of *E. coli*, but not any of the other genera (100% exclusivity), and all strains of *E. coli* included in the study tested positive (100% inclusivity). The LOD_{50} for *E. coli* with the *phoA* gene was 2.8 log CFU g^{-1} in both artificially inoculated beef and bovine faeces. When applied to both beef and faeces, the LOD_{50} for *E. coli* with the *stx1* target was 3.2 log CFU g^{-1}. However, the LOD_{50} for *E. coli* with the *stx2* target differed and was 3.5 and 2.8 log CFU g^{-1} for beef and faeces, respectively; perhaps suggesting that *stx2* amplification using the primers selected for our LAMP assay may be more affected by matrix inhibitors than the *stx1* primers selected.

LAMP assay positive samples can be detected by gel electrophoresis, endpoint visual observation of colour, or turbidity changes. In this study real-time monitoring of fluorescence signals was possible on the Genie II instrument, which made it possible to quantitatively detect the specific targets. In the present LAMP method, the R^2 values, for *E. coli* numbers ranging from 10^3–10^7 CFU g^{-1} were found to be 0.963-0.989 and 0.763-0.932 for beef and faeces samples, respectively. The superior quantitative capability observed for beef compared to faeces could be attributable to the nature of two sample matrices; the latter may have contained larger amounts of inhibitors (Opel et al., 2010; Lawal et al. 2015).

To the best of our knowledge, this is the first study that investigated the multiplex LAMP detection of *E. coli* and VTEC in beef and bovine faeces. Dong et al. (2014) developed a LAMP assay for the simultaneous detection of only VTEC *stx1* and *stx2* genes in bovine faeces and environmental samples. However, the detection limit was determined

on a DNA concentration basis rather than CFU and thus results are not comparable. Individual LAMP assays, each targeting the main VTEC serogroups (O26, O45, O103, O111, O121, O145, and O157) have also been developed (Wang et al. 2012a). These assays were specific and able to detect 10^3-10^4 CFU g^{-1} in artificially contaminated lettuce, spinach, minced beef and beef trimmings) and were quantitative (R^2 =0.867 to 0.999 compared to culture counts). When an enrichment step (6-8 h) was included the assays were able to detect lower levels of VTEC (1-2 and 10-20 CFU 25 g^{-1}). Serogroup-independent LAMP assays for the detection of VTECs by individually targeting the *stx1, stx2*, and *eae* genes have also been developed for minced beef meat (Wang et al. 2012b). These were shown to be specific and sensitive, achieving detection limits of 1-20 CFU $reaction^{-1}$ in pure culture and 10^3-10^4 CFU g^{-1} in artificially inoculated minced beef, as well as showing good quantitative capabilities (R^2=0.904-0.997 compared to culture counts). The authors also found that when an enrichment step was incorporated (6-8 h), the assays accurately detected even lower levels of VTECs in beef (1-2 and 10-20 CFU 25 g^{-1}). Application of these assays to test human stools showed that they were able to detect VTECs artificially inoculated at 10^3 or 10^4 CFU 0.5 g^{-1} stool after 4 h enrichment.

Molecular-based detection methods (such as PCR and LAMP) encounter various inhibitors when used to test different biological matrices (Wilson 1997; Schrader et al. 2012). However, it is claimed that LAMP is more robust than PCR in terms of its susceptibility to amplification inhibitors (Kaneko et al. 2007; Wang et al. 2012a), which could potentially permit the application of the LAMP assay without the requirement for DNA purification or culture enrichment. A LAMP assay detection limit of $4.1x10^4$ CFU ml^{-1} for detection of *E. coli* O157 in raw milk, without the incorporation of an enrichment step in the procedure, has previously been reported (Wang et al. 2009). Romero et al. (2015) developed a rapid LAMP assay that was able to detect thermotolerant *Campylobacter* spp. in boot swab samples, without the use of culture enrichment or DNA extraction. It was shown to have a detection limit of 10^4 CFU ml^{-1}, making on-site use a possibility. Ravan et al. (2016) developed a LAMP assay that targets a highly specific region of the Z3276 gene for the detection of *E.*

coli O157:H7 in artificially contaminated ground beef slurry with a sensitivity level of 10^3 CFU ml^{-1} in the absence of an enrichment step. Several studies have shown that LAMP detection sensitivity is similar to or greater than that of PCR (Okamura et al. 2008; 2009; Wang et al. 2012a).

The multiplex LAMP assay developed during this study was demonstrated to have a LOD_{50} in the range 2.8-3.5 log CFU g^{-1} beef for *E. coli* generically and VTEC specifically. In contrast, the comparator pour-plate method for enumerating *E. coli* in beef used by the industry has a detection limit of 1.0 log CFU g^{-1} beef; testing of beef for presence of VTEC specifically is not currently carried out. Clearly, the culture method has greater detection sensitivity for generic *E. coli* than the novel LAMP assay, and, given that 10–100 CFU VTEC can cause illness (Teunis et al. 2004), the latter would also have insufficient detection sensitivity, in its present form, to be of use for VTEC monitoring within the beef industry. The results from the validation experiments conducted with retail beef and bovine faeces show that when moderate to high levels of *E. coli* (>10^2-10^3 CFU g^{-1}) were present there was good correlation between the counts obtained by conventional culture and the predicted *E. coli* counts obtained via the LAMP method. Lawal et al. (2015) reported similar R^2 values (0.86-0.88) when validating a new VTEC PCR detection method in comparison with conventional enumeration for testing bovine recto-anal swabs. False negative LAMP assay results are suggested by the 12.5 and 14.7% of beef and faeces samples, respectively, that tested culture positive but LAMP assay negative for all three *E. coli* gene targets. Given that no VTEC were detected by the LAMP assay in any of the naturally contaminated beef or faeces samples, and since the culture method employed does not distinguish between non-VTEC and VTEC, the VTEC part of the novel LAMP assay remains to be fully validated. In order to improve LAMP detection sensitivity, an enrichment step prior to LAMP would be a possibility, as has been demonstrated in previous studies by Wang et al. (2012a), or perhaps a touchdown LAMP approach could be investigated, as was reported recently by Wang et al. (2015). However, it should be noted that after an enrichment step quantification of *E. coli* or VTEC would not be possible any more.

In conclusion, a multiplex LAMP assay was successfully developed and optimised during the course of this proof-of-concept study. The novel LAMP assay is able to detect and distinguish between generic *E. coli* and VTEC when present on beef or in bovine faeces at levels of $>10^2$-10^3 CFU g^{-1}, as demonstrated by the results of testing of artificially spiked samples. The same has still to be demonstrated for naturally contaminated beef or bovine faeces samples, since no samples positive for VTEC were encountered during the small-scale testing carried out to date. Currently, the detection sensitivity of the novel multiplex LAMP assay (10^2-10^3 CFU g^{-1}) may be sufficient for it to be used for monitoring the hygienic status of beef, to ensure compliance with microbiological criteria on levels of *E. coli* in beef. Subsequent to some further development to improve detection sensitivity, the novel multiplex LAMP assay could potentially represent a serogroup-independent method of quickly detecting VTEC strains in beef or bovine faeces samples.

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Conflict of interest

Author Steve Millington is employed by OptiGene Limited who supplied the Genie II real-time fluorometer instrument used in this study. He assisted with LAMP primer design during this study. Other authors declare no conflict of interest.

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Table 1. Bacterial species and strains used in this study to check specificity of *phoA* LAMP primers.

Bacterial cultures[a]	**Number of strains**	***phoA* LAMP assay result**
Escherichia coli (ATCC BAA 1427, BAA 1428 and BAA 1429)	3	+
E. coli K12 NCTC 10358	1	+
E. coli ATCC 43894, serotype: O157:H7	1	+
E. coli PHL09, serotype O157:H7[b]	1	+
E. coli VSD 397, serotype: O157:H7[b]	1	+
E. coli EDL 933, serotype: O157:H7[b]	1	+
E. coli CDC 99-311, serotype: O145[b]	1	+
E. coli CDC 03-3014*, serotype:* O26:H11[b]	1	+
E. coli NCTC 12900	1	+
E. coli ER 2738[c]	1	+
Bacillus subtilis[b]	1	-
B. cereus NCTC 11145	1	-
Campylobacter lari NCTC 11458	1	-
Campylobacter coli ATCC 43478	1	-
Campylobacter jejuni ATCC 29428	1	-
Listeria innocua NCTC 11288	1	-
L. innocua CM 235[b]	1	-
Listeria monocytogenes (NCTC 4855, 1/2a[b], 1/2b[b], CM 006[b], CM 109[b], CM 097[b], CM 092[b], CM 093[b],	10	-

CM 108 [b], CM 191 [b])		
L. grayi ATCC 19120	1	-
L. murrayi NCTC 10812	1	-
L. welshimeri (CM 100 [b], CM 109 [b])	2	-
Salmonella Reading [b]	1	-
S. Senftenberg [b]	1	-
S. Livingstone [b]	1	-
S. Infantis [b]	1	-
S. Montenegro [b]	1	-
S. Kottbus [b]	1	-
S. Tennessee [b]	1	-
S. Rissen [b]	1	-
S. Menston [b]	1	-
S. Virchow [b]	1	-
S. Orion [b]	1	-
S. Newport [b]	1	-
S. Kentucky [b]	1	-
S. London [b]	1	-
S. Typhimurium [b]	3	-
S. Enteritidis NCTC 6676 [b]	1	-
Staphylococcus aureus [b]	1	-
Cronobacter malonaticus E833	1	-
Cronobacter sakazakii ATCC 12868	1	-
Cronobacter muytjensii ATCC 51329	1	-
Mycobacterium	1	-
avium subsp. *paratuberculosis*		
ATCC 19698		

Micrococcus sp.[b]	1	-
Enterobacter sp.[b]	1	-
Total	58	

[a] Origin of cultures: NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; [b] Agri-Food and Biosciences Institute for Northern Ireland (Newforge Lane, Belfast) culture collection; [c] New England Biolabs.

Table 2. LAMP primers used in the present study to specifically target *phoA*, *stx1* and *stx2* genes of *E. coli.*

Primer name	Primer sequence	Reference
Ec phoA F3	AAGTTGAAGGTGCGTCAAT	This study
Ec phoA B3	CTTGTGAATCCTCTTCGGAG	
Ec phoA FIP	GTGATCAGCGGTGACTATGACCTCTCGATGAAGCCGT ACA	
Ec phoA BIP	ATTGTCGCGCCGGATACCCTCATCACCATCACTGCG	
Ec phoA LoopF	AGCGTGTTGCCATCCTTT	
Ec phoA LoopB	CAGGCGCTAAATACCAAAGATG	
Ec stx1 F3	ACAACAGCGGTTACATTGT	Dong et al.
Ec stx1 B3	GATCATCCAGTGTTGTACGAA	(2014)
Ec stx1 FIP	GCGATTTATCTGCATCCCCGTATGTCTGGTGACAGTAG CTAT	
Ec stx1 BIP	GGAACCTCACTGACGCAGTCCTTCAGCTGTCACAGTAA CA	
Ec stx1 LoopF	ACTGATCCCTGCAACACG	
Ec stx1 LoopB	TGTGGCAAGAGCGATGTT	
Ec stx2 F3	GCATCCAGAGCAGTTCTG	Dong et al.
Ec stx2 B3	CAGTATAACGGCCACAGTC	(2014)
Ec stx2 FIP	GGCGTCATCGTATACACAGGAGCGCTTCAGGCAGATA CAG	
Ec stx2 BIP	AGACGTGGACCTCACTCTGAAACTCTGACACCATCCTC TC	
Ec stx2 LoopF	CAGACAGTGCCTGACGAA	
Ec stx2 LoopB	GGCGAATCAGCAATGTGC	

Table 3. LAMP assay detection limits by gene target, determined using pure *E. coli* cultures (non-pathogenic and VTEC) tested individually.

E. coli strain or serotype	Gene targets present	Limit of detection (LOD_{50}) by target gene (log CFU ml^{-1})		
		phoA	Stx1	Stx2
NCTC 12900	*phoA*	2.5	-	-
BAA 1427	*phoA*	3.5	-	-
BAA 1428	*phoA*	3.5	-	-
BAA 1429	*phoA*	2.5	-	-
ER 2738	*phoA*	3.5	-	-
K12	*phoA*	2.5	-	-
VSD397 O157	*phoA, stx2*	2.9	-	3.5
EDL 293 O157	*phoA, stx1, stx2*	2.5	2.8	2.8
CDC 03-014 O26:H11	*phoA, stx1, stx2*	2.8	2.5	2.5
CDC 9-3311-O145	*phoA, stx1, stx2*	3.5	3.2	3.5

Table 4. Limit of detection 50% (LOD_{50}), and associated 95% confidence limits, of the optimised multiplex LAMP assay for detection of *E. coli*, determined by testing triplicate samples of beef and bovine faeces artificially inoculated with different concentrations of *E. coli* (non-VTEC and VTEC).

Sample matrix	Gene target	No. of samples positive per spiking level (CFU g^{-1})						LOD 50% (log CFU g^{-1})	95% confidence limits (log CFU g^{-1})
		10^7	10^6	10^5	10^4	10^3	10^2		
Beef	*phoA*	3/3	3/3	3/3	3/3	4/6*	0/3	2.8	2.4-3.3
	Stx1	3/3	3/3	3/3	3/3	0/3	0/3	3.2	2.5-3.9
	Stx2	3/3	3/3	3/3	3/3	3/3	0/3	3.5	3.5-3.5
Faeces	*phoA*	3/3	3/3	3/3	3/3	4/6*	0/3	2.8	2.4-3.3
	Stx1	3/3	3/3	3/3	3/3	3/3	0/3	3.2	2.5-3.9
	Stx2	3/3	3/3	3/3	3/3	3/3	0/3	2.8	2.1-3.5

*Results for artificial inoculations of both non-VTECS and VTECs were combined.

Table 5. Quantification equations and correlation coefficient (R^2) values obtained from calibration curves generated with the LAMP assay applied to beef and bovine faeces artificially spiked with *E. coli* (non-VTEC and VTEC).

Sample matrix	Gene target	Quantification equation	R^2
Beef	*phoA*	y= -2.5897x+28.219	0.989
	Stx1	y= -2.1748x+24.348	0.932
	Stx2	y= -2.1221x+25.484	0.963
Faeces	*phoA*	y= -2.5912x + 28.068	0.932
	Stx1	y= -2.3509x + 25.479	0.955
	Stx2	y= -3.1648x + 29.088	0.763

Figure 1. Examples of the peaks obtained after annealing curve analysis of the different primer sets. Peaks from left to right correspond to *phoA* (A) primer sets c, d, e and b, respectively. Peaks from left to right correspond to stx1 primer set A, *stx1* primer set B, *stx2* primer set B, *stx2* primer set A, respectively (B).

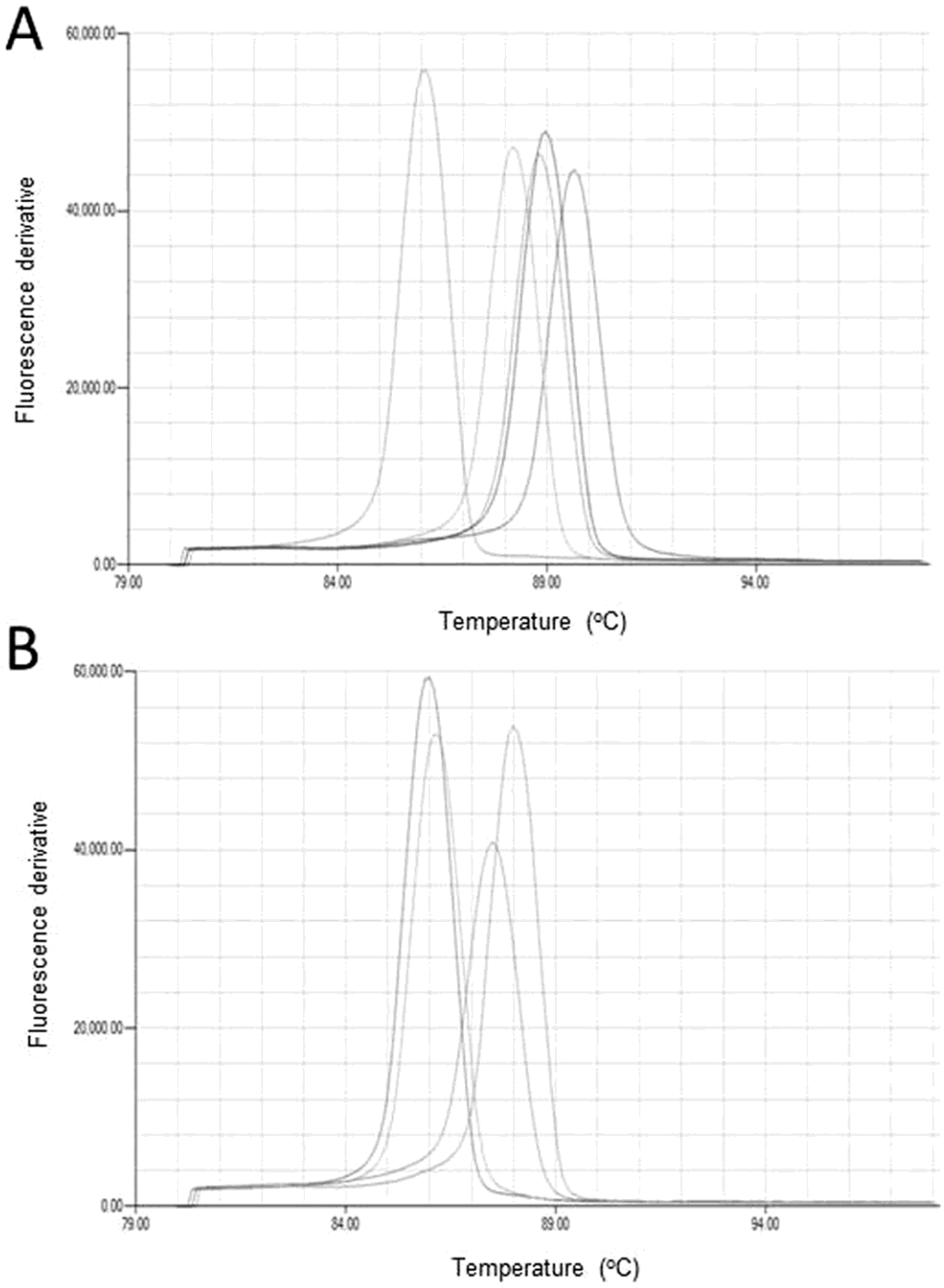

Figure 2. Typical peaks generated after annealing curve analysis when the optimised LAMP assay was applied to beef inoculated with non-VTEC *E. coli* strain(s) (A) and VTEC strain(s) harbouring both toxin producing genes (B). The peaks represent the *Tm* values of each of the three gene targets. For B, from left to right, peaks correspond to *stx1*, *stx2* and *phoA* genes. Samples positive for *E. coli* only will appear as Fig.2A and a sample positive for VTEC will appear as Fig.2B.

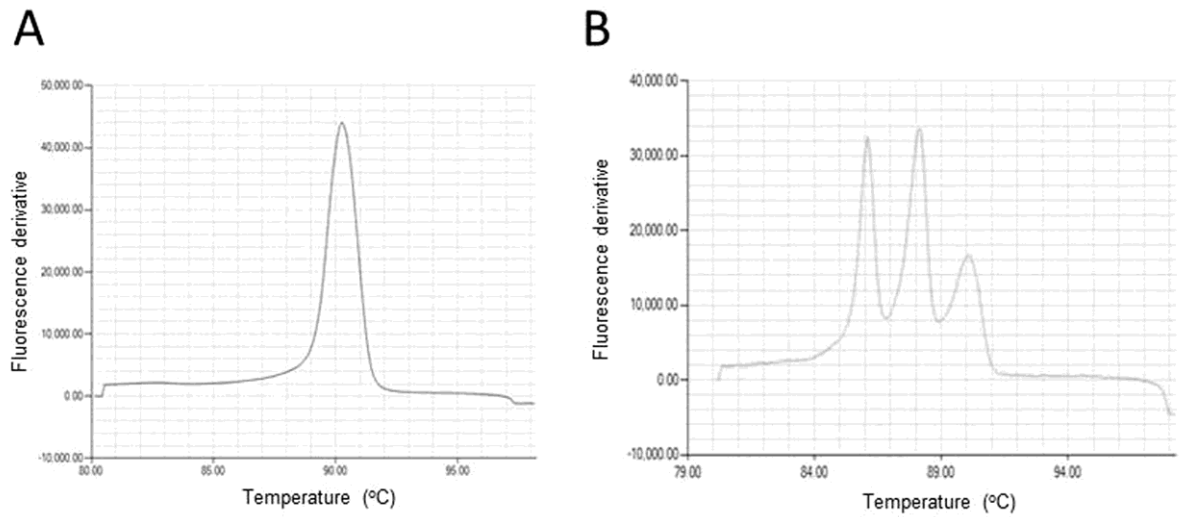

Figure 3. (A) Typical LAMP amplification curves for dilutions of artificially inoculated beef containing decreasing numbers of *E. coli* (curves from left to right 10^7, 10^6, 10^5, 10^4, 10^3, CFU g^{-1}) (B) Corresponding calibration curve for the *phoA* gene. Each point in (B) is the mean of three replicates. No amplification was detected for *E. coli* levels lower than 10^3 CFU g^{-1} beef.

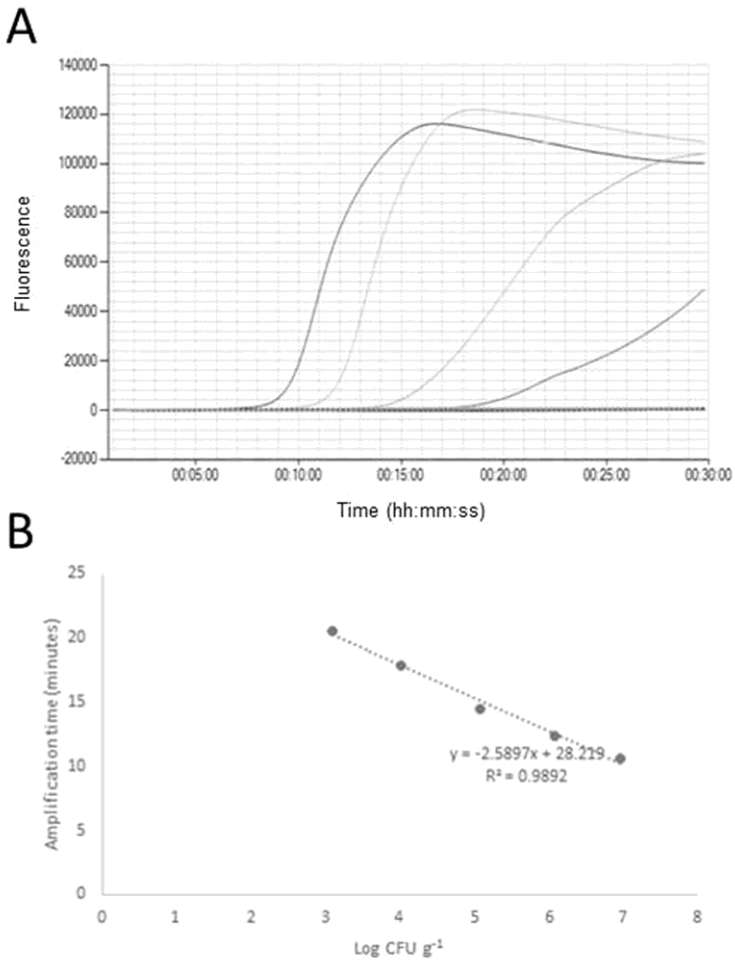

Figure 4. Correlation between the *E. coli* count predicted by the LAMP *phoA* assay (using the equation of the regression line for artificially contaminated bovine faeces, see Table 4) and conventional *E. coli* count on Rapid 2 agar obtained for 17 *E. coli* positive naturally contaminated bovine faeces samples.

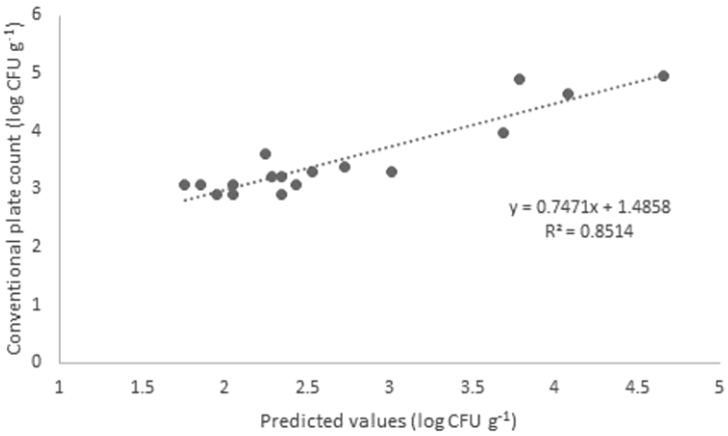

Figure 5. Correlation between the *E. coli* count predicted by the LAMP *phoA* assay (using the equation of the regression line for artificially contaminated beef, see Table 4) and conventional *E. coli* counts on Rapid 2 agar obtained for four *E. coli* positive naturally contaminated retail beef samples.